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Plant litter enhances degradation of the herbicide MCPA and increases formation of biogenic non-extractable residues in soil

Karolina M. Nowak[a,b,*], Anja Miltner[b], Christian Poll[c], Ellen Kandeler[c], Thilo Streck[d], Holger Pagel[d]

[a] Technische Universität Berlin, Institute of Biotechnology, Chair of Geobiotechnology, Ackerstraße 76, 13355 Berlin, Germany
[b] Helmholtz-Centre for Environmental Research – UFZ, Department of Environmental Biotechnology, Permoserstr. 15, 04318 Leipzig, Germany
[c] University of Hohenheim, Institute of Soil Science and Land Evaluation, Department of Soil Biology, Emil-Wolff-Str. 27, 70599 Stuttgart, Germany
[d] University of Hohenheim, Institute of Soil Science and Land Evaluation, Department of Biogeophysics, Emil-Wolff-Str. 27, 70599 Stuttgart, Germany

ABSTRACT

Amendment of soils with plant residues is common practice for improving soil quality. In addition to stimulated microbial activity, the supply of fresh soluble organic (C) from litter may accelerate the microbial degradation of chemicals in soils. Therefore, the aim of this study was to test whether the maize litter enhances degradation of 4-chloro-2-methylphenoxyacetic acid (MCPA) and increases formation of non-toxic biogenic non-extractable residues (bioNERs). Soil was amended with $^{13}C_6$-MCPA and incubated with or without litter addition on the top. Three soil layers were sampled with increasing distance from the top: 0–2 mm, 2–5 mm and 5–20 mm; and the mass balance of $^{13}C_6$-MCPA transformation determined.

Maize litter promoted microbial activity, mineralization of $^{13}C_6$-MCPA and bioNER formation in the upper two layers (0–2 and 2–5 mm). The mineralization of $^{13}C_6$-MCPA in soil with litter increased to 27% compared to only 6% in the control. Accordingly, maize addition reduced the amount of extractable residual MCPA in soil from 77% (control) to 35% of initially applied $^{13}C_6$-MCPA. While non-extractable residues (NERs) were < 6% in control soil, litter addition raised NERs to 21%. Thereby, bioNERs comprised 14% of $^{13}C_6$-MCPA equivalents. We found characteristic differences of bioNER formation with distance to litter. While total NERs in soil at a distance of 2–5 mm were mostly identified as ^{13}C-bioNERs (97%), only 45–46% of total NERs were assigned to bioNERs in the 0–2 and 5–20 mm layers. Phospholipid fatty acid analysis indicated that fungi and Gram-negative bacteria were mainly involved in MCPA degradation. Maize-C particularly stimulated fungal activity in the adjacent soil, which presumably facilitated non-biogenic NER formation. The plant litter accelerated formation of both non-toxic bioNERs and non-biogenic NERs. More studies on the structural composition of non-biogenic NERs with toxicity potential are needed for future recommendations on litter addition in agriculture.

1. Introduction

Microbial degradation ultimately degrades pesticides in soil (Kästner et al., 2014) and results in the formation of transformation products, microbial biomass, mineralization products and non-extractable residues (NERs; Barriuso et al., 2008). NERs in soils are considered as a 'black box' since their structural identity in most cases is unexplained due to the complexity of the soil matrix and to the associated analytical challenges (Barriuso et al., 2008; Kästner et al., 2014). Special care is given to non-biogenic NERs which may contain the toxic parent pesticide or its transformation products (Barriuso et al., 2008; Kästner et al., 2014). The parent pesticide and/or its transformation

products can be physically sequestered within solid matrix (as NERs type I) or covalently bound to organic matter (OM) of soil (as NERs type II) (Barriuso et al., 1997; Barriuso et al., 2008; Kästner et al., 2014). Physically sequestered chemicals can be remobilized from soil after freeze-thawing or heavy rain events causing delayed environmental pollution (Barriuso et al., 2008; Kästner et al., 2014). However, NERs from several ^{13}C-labeled pesticides, e.g. dichlorophenoxyacetic acid (2,4-D) (Nowak et al., 2011), metamitron (Wang et al., 2017) and glyphosate (Muskus et al., 2019; Muskus et al., 2020) in soil could be largely assigned to the non-toxic biogenic NERs (bioNERs). The bioNERs are formed by assimilation of pesticide-derived carbon (C) into the microbial biomass, e.g. fatty acids (FAs) and amino acids (AAs), and

stabilization of biomass residues in non-living OM after microbial death (Nowak et al., 2011; Wang et al., 2017).

The addition of plant residues to agricultural topsoils is common farming practice which improves soil physico-chemical properties, provides habitats to mesofauna, and prevents soils from erosion (López-Piñeiro et al., 2013; Wild et al., 2014). The soluble organic C and nutrients from decomposing plant residues (so-called 'detritusphere') promote microbial growth and activity as well as pesticide biodegradation (Poll et al., 2010). Therefore, the plant residue may also reduce the environmental risk of applied pesticides by promoting faster mineralization and bioNER formation (Ghani and Wardle, 2001; Shaw and Burns, 2003; Gerhardt et al., 2009; Ditterich et al., 2013; Pagel et al., 2016; Saleh et al., 2016).

4-chloro-2-methylphenoxyacetic acid (MCPA) represents the phenoxyacetic acid class of pesticides (Zaprasis et al., 2010). MCPA is commonly used herbicide and is the third top non-agriculture pesticide on the list of pesticide sales (US EPA, 2017). In addition, the degradation pathways of MCPA are well described and this pesticide is structurally similar to other phenoxyacetic acid herbicide 2,4-D from which major formation of bioNERs has been documented (Nowak et al., 2011). Poll et al. (2010) have already shown that soluble C from the maize litter on topsoil promoted microbial conversion of ^{14}C-MCPA to $^{14}CO_2$ in soil. This suggests that maize litter-C input might trigger accelerated NER formation as the consequence of stimulated microbial activity in the detritusphere. Maize litter was selected as a model plant residue, whereas MCPA was chosen as a model compound to test the effect of plant residue on the extent of bioNER or non-biogenic NER formation during pesticide degradation in soil.

Our study aimed to clarify the influence of soluble C from decomposing plant residues (maize litter) on MCPA degradation and on NER formation in soil. We hypothesized that stimulated microbial activity promotes bioNER formation in the upper layers of soil and that the formation of NERs differs at a fine mm-depth scale. This was tested in soil microcosms without (as a control setting) and with litter addition on top, sampled in three layers: 0–2, 2–5 and 5–20 mm. The microcosm approach allowed the detection of small-scale gradients under controlled conditions which would not be possible in the field with many interfering environmental factors. The general mass balance of $^{13}C_6$-MCPA turnover (mineralization, extractable and NERs) was quantified. The quantitation also included ^{13}C incorporation into microbial biomass based on analyses of FAs and AAs as biomarkers for living biomass and biomass residues in soil.

2. Material and methods

2.1. Chemicals

All chemicals used were of the highest quality commercially available. Unlabeled MCPA was obtained from Sigma Aldrich (PESTANAL®). $^{13}C_6$-MCPA (> 99 at% ^{13}C and chemical purity) was obtained from IsoSciences (USA).

2.2. Soil and plant residues

We used topsoil from a loamy Luvisol (WRB 2006) from an agricultural field at the research station Scheyern (Germany; 48°30′N, 11°21′E; plot "A18", Helmholtz Zentrum München). The soil was sampled, sieved (< 2 mm) and stored in the dark at −20 °C. Before use the soil was thawed and its initial gravimetric water content of 27% was reduced to 18% by air drying at 20 °C during an acclimatization period of 20 days in the dark. The acclimatized soil was then immediately used in the microcosm experiment. In this study, we prepared a one-to-one mixture of chopped maize leaves and stems (2–10 mm long and 1–3 mm thick) and spread it on top of the soil surface (see Section 2.3 for details of the experimental setup; see also Photo S1). Chemical soil and litter properties are compiled in Table 1.

2.3. Experimental design

Microbial turnover of $^{13}C_6$-MCPA, including bioNER formation in agricultural soil amended with maize litter was investigated in repacked soil columns of 30 mm height. We set up two experimental treatments: (1) soil spiked with MCPA and without litter (control) and (2) soil spiked with MCPA and amended with litter (litter). In order to account for the natural ^{13}C abundance in soil and litter, both treatments were spiked either with unlabeled MCPA (reference) or $^{13}C_6$-MCPA. Two spiking solutions were prepared by dissolution of i) unlabeled MCPA, ii) ^{13}C-labeled MCPA in distilled water; both solutions were adjusted to pH 5.3 with 0.1 M NaOH. These solutions were then separately mixed with soil to obtain a final MCPA concentration of 50 mg kg^{-1} of soil dry weight (total MCPA-C: 2.243 μmol g^{-1}, ^{13}C: 1.495 μmol g^{-1}) and a gravimetric water content of 28%. Portions of MCPA-amended soil were filled to a height of 30 mm into stainless steel cylinders (diameter 56 mm, height 40 mm) and compacted to a bulk density of 1.2 g cm^{-3}. A mass of 0.5 g of maize residues (rewetted with 2 mL 0.01 M CaCl$_2$) was added on top of the soil cores for the litter treatment. The soil cores were placed separately in airtight microcosms (glass containers, 750 mL) and incubated at 20 °C in the dark for 65 days (litter) and for 77 days (control). Microcosms were flushed every 2–3 days with fresh ambient air to maintain sufficient oxygen supply. Water losses from soil cores were determined by regular weighing and found to be negligible. Each treatment (unlabeled and labeled MCPA versions for control and litter amendment) was replicated six times. For detailed information on the experimental set-up and the number of replicates taken for the respective analysis please refer to the Scheme S1 in the Supplementary Information.

2.4. Sample preparation

After removing the litter layer (which was not analyzed), the soil cores amended with unlabeled MCPA and $^{13}C_6$-MCPA were immediately frozen at −20 °C. They were then sliced using a cryostat microtome (HM 500 M, MICROM International GmbH, Walldorf, Germany) into the following layers: 0–2 mm (detritusphere), 2–5 mm (transition zone) and 5–20 mm (bulk soil) from top of mineral soil. Cores without litter were sampled in the same way, although a detritusphere was obviously absent. Therefore, in results and discussion sections, for the control we refer to the specific depth (0–2, 2–5 or 5–20 mm), whereas for the litter treatment 0–2 mm is termed as 'detritusphere', 2–5 mm as 'transition zone', and 5–20 mm as 'bulk soil'. To obtain sufficient material for analyses (in particular for the two upper layers 0–2 and 2–5 mm), we combined and homogeneously mixed the soil from the associated layers of two soil cores into one. This procedure yielded three samples (0–2, 2–5 and 5–20 mm) per treatment and layer derived from six replicate soil cores.

2.5. Respiration and soil organic carbon

CO$_2$-C production (total C and ^{13}C) from the whole soil column was measured during the experiment at regular intervals (2–3 days) by trapping the evolved CO$_2$ in the headspace of the microcosms in 1 M NaOH solution. Sampling of NaOH and analysis of total CO$_2$ and $^{13}CO_2$ as well as measurements of amount and ^{13}C abundance of soil organic carbon were done as described in Poll et al. (2008; 2010). Soil cores were sampled when both soil respiration and MCPA mineralization reached constant rates because we did not expect significant changes in the fate of the compound thereafter. This was the case after 65 days for the cores with litter amendment and after 77 days for the cores without litter amendment (control). Although the latter were incubated for a longer time both soil respiration and MCPA mineralization were lower in the absence of litter than in the cores with litter amendment.

Table 1
Basic characteristics of soil and corn litter. Number in parentheses represent standard errors (n = 3).

	Total C mg g^{-1}	Total N mg g^{-1}	δ^{13}C ‰	pH (CaCl$_2$)	sand %	silt %	clay %
Soil	14.9 (0.4)	1.85 (0.02)	−25.9 (0.04)	5.3	19 (0.4)	63 (0.5)	17 (0.1)
Corn litter	412 (5)	8.81 (0.20)	−12.8 (0.01)	n.d.[1]	n.a.[2]	n.a.	n.a.

[1] n.d. not determined.
[2] n.a. not applicable.

2.6. MCPA

Residual MCPA in soil was extracted by shaking 1.5 g soil with 7.5 mL methanol/H$_2$O (1:1 by volume) in 15 mL centrifuge tubes (polyethylene) on a horizontal shaker at 200 rpm for 10 min. The tubes were then heated in a water bath to 50 °C for 60 min. After that, shaking on the horizontal shaker (200 rpm for 10 min) was repeated and the samples centrifuged (4500g, 10 min). 1.5 mL aliquots of the supernatant were filtered (0.45 μm syringe filters, regenerated cellulose) directly into HPLC vials for further analysis. MCPA in the extracts was determined by HPLC with a UV-detector (System Gold, Beckman Instruments) at a wavelength of 228 nm using acetonitrile/water (ratio 32:68) with 20 mmol L^{-1} H$_3$PO$_4$ as mobile phase at a flow rate of 0.5 mL min^{-1} on a 3 μm MZ Aqua Perfect C18 material column (150 mm × 3 mm; MZ-Analysentechnik GmbH, Germany). Identification and quantification was done by external calibration using freshly prepared MCPA standards dissolved in methanol/H$_2$O (1:1 by volume). The detection limit of MCPA in soil was 0.05 μg g^{-1}, and the recovery of MCPA from freshly spiked soil samples was 96%.

2.7. Fatty acids (FAs)

Incorporation of ^{13}C-derived MCPA into microbial lipids was analyzed using phospholipid fatty acids (PLFAs) and in total FAs (tFAs). PLFAs are used to assess the amount of living microbial biomass *in situ* (Zelles, 1999; Kaur et al., 2005). The tFAs, which include the PLFAs and the FAs in the non-living OM (FAs$_{OM}$) are indicating the stabilization of FAs in the OM (Drenovsky et al., 2004; Nowak et al., 2011). The PLFAs and FAs$_{OM}$ thus provide information about presence of living and non-living groups of microorganisms *in situ*, respectively (Zelles, 1999; Kaur et al., 2005). FAs biomarkers are indicative of microbial groups: (i) Gram-positive bacteria (iso- and anteiso-branched FAs), (ii) actinomycetes (Gram-positive phylum, 10-methyl branched FAs), (iii) Gram-negative bacteria (monounsaturated FAs) and (iv) fungi + eukaryotes (polyunsaturated FAs). Starvation of Gram-negative bacteria is indicated by the presence of cyclopropyl FA (Kaur et al., 2005). Saturated straight chain FAs are ubiquitous and thus cannot be assigned to any specific group. They are not explicitly considered in the present study but are included in the total contents.

The detailed extraction, purification and derivatization procedures were described previously (Nowak et al., 2011). Briefly, PLFAs were extracted from soil with phosphate buffer/methanol/chloroform (Bligh and Dyer; 1959) and separated from neutral lipids and glycolipids over a silica gel column (Unisil, Clarkson Chromatography Products, South Williamsport, USA; Nowak et al., 2011). The PLFAs were then derivatized using methanol/trimethylchlorosilane (9:1; v:v; Miltner et al., 2004). The tFAs were directly derivatized in the same way as the PLFAs and the methylated tFAs were extracted from soil with diethyl ether and purified over silica gel columns (Mallinckrodt Baker Germany, Griesheim, Germany; Miltner et al., 2004). The derivatized fatty acid methyl esters (FAME) in both fractions were separated on a BPX-5 column (30 m × 0.25 mm × 0.25 μm) and identified and quantified by means of gas chromatography-mass spectrometry (GC–MS). The isotopic composition of FAME was determined by gas chromatography–combustion– isotope ratio mass spectrometry (GC–C–irMS).

The exact analytical conditions for both chromatographic devices were described previously (Nowak et al., 2011).

2.8. Amino acids (AAs)

In analogy to FAs, the incorporation of ^{13}C-derived MCPA into amino acids (AAs) was analyzed in the living biomass fraction (biomass AA; bioAAs) and in the total AA fraction (tAAs) containing both bioAAs and AAs stabilized in OM (AAs$_{OM}$). Unlike to FAs, AAs biomarkers do not provide any phylogenetic information. However, they are very important due to their high stability and highest abundance in microbial cells (Nowak et al., 2011). Based on the measured ^{13}C in the AAs, the total amount of bioNERs can be estimated considering a 50% abundance of proteins in the cell (for details see Section 2.9).

Total AAs (tAAs) were hydrolyzed from proteins with 6 M HCl and purified over cation exchange resin (DOWEX 50 W-X8, 50–100 mesh) as described previously by Nowak et al. (2011). After purification, the carboxyl groups of AAs were isopropylated and the amino groups trifluoroacetylated (Silfer et al., 1991; Miltner et al., 2009). The remaining impurities after first purification and derivatization were extracted into phosphate buffer (Ueda et al., 1989; Nowak et al., 2011). In the case of bioAAs, the biomass was first extracted from the soil with Amberlite IRC-748 and sodium deoxycholate/polyethylenglycol mixture (Miltner et al., 2009). Thereafter, biomass pellets containing AAs were further hydrolyzed, purified and derivatized as described above. The identity, quantity and isotopic composition of the bioAAs and tAAs were determined by means of GC–MS and GC–C–irMS using the same instruments and columns as for FAs analysis. All the analytical conditions for AAs separation using GC–MS and GC–C–irMS were described previously (Nowak et al., 2011).

The recovery of microbial biomass from soil is low, two independent studies with different soils showed that the recovery of microbial biomass was about 40% (Jacobsen and Rasmussen, 1992; Miltner et al., 2009). Therefore, the bioAAs data are underestimated and we present the original bioAAs data in Section 3. Results. For estimating the AAs in the non-living OM (AAs$_{OM}$), values for AAs in living biomass were extrapolated from the measured values based on an assumed 40% extraction efficiency for bioAAs.

2.9. Calculation of MCPA-derived ^{13}C in CO$_2$, FAs, AAs and biogenic NER

The isotopic compositions of tAAs, bioAAs, PLFAs and tFAs were corrected for C shifts due to derivatization (Silfer et al., 1991; Boschker, 2004).

We calculated MCPA-derived ^{13}C ($^{13}C_{MCPA}$) in CO$_2$, soil, FAs and AAs based on total C and ^{13}C mass balances as follows:

$$C_t = C_{soil} + C_{MCPA}^{sc} + C_{MCPA}^{r} = C_{bulk} + \,^{13}C \tag{1}$$

$$^{13}C_t = \,^{13}F_t \cdot C_t = F_{ref} \cdot C_{bulk} + \,^{13}C_{MCPA} \tag{2}$$

Combining Eqs. (1) and (2) leads to:

$$^{13}C_{MCPA} = \frac{^{13}C_t - F_{ref} \cdot C_t}{1 - F_{ref}} = \frac{^{13}F_t - F_{ref}}{1 - F_{ref}} \cdot C_t \tag{3}$$

Here, C_t and $^{13}C_t$ stand for the total amount of C and ^{13}C in CO$_2$, soil or

fatty acids. C_{bulk} denotes the amount of C derived from soil organic carbon (C_{soil}) and non-labeled side chain MCPA

(C_{MCPA}^{sc}) and C_{MCPA}^{r} is the amount of ring MCPA-C. The values $^{13}F_t$ and F_{ref} stand for the molar $\frac{^{13}C}{^{12}C + ^{13}C}$ ratios in CO_2, soil, FA or AA from $^{13}C_6$-MCPA-amended and reference samples, respectively.

The amounts of ^{13}C in CO_2, soil, PLFAs, tFAs, bioAAs and tAAs are given as percentages of the initially applied $^{13}C_6$-MCPA. The calculation of total amounts of bioNERs formed during biodegradation of $^{13}C_6$-MCPA was based on the measured content of ^{13}C-tAAs. AAs hydrolyzed from proteins are the major constituents of microbial biomass (50% of the total biomass carbon; Nowak et al., 2011). Therefore, a conversion factor of two was used for estimation of the total bioNERs from ^{13}C-tAAs concentrations.

The respiration, mineralization and ^{13}C mass balance of $^{13}C_6$-MCPA are shown as integrated data (comprising the three layers) over the whole soil column for control and maize litter amended soil. The FAs, AAs and the extractable $^{13}C_6$-MCPA were measured at each different soil layer (0–2, 2–5 and 5–20 mm) and thus those data for control and litter amended soil are shown for each layer separately.

2.10. Statistics

Mean and standard deviation of calculated $^{13}C_{MCPA}$ values were estimated by uncertainty propagation using second-order Taylor expansion of Eq. (3) combined with Monte Carlo simulation (Taylor, 1997; JCGM, 2008) for generating samples from a multivariate normal distribution based on means and standard deviations of measured C_t, $^{13}F_t$ and F_{ref} values. This was done using the package 'propagate' (Spiess, 2014) with the statistical software 'R' (R Core Team, 2015).

We used Welch's *t*-test as implemented in the R package 'BSDA' (Arnholt, 2012) to compare soil respiration, MCPA mineralization, total MCPA-derived C, ^{13}C-PLFAs, ^{13}C-tFAs, ^{13}C-bioAAs and ^{13}C-tAAs of soil cores with and without litter amendment at individual sampling times and depths, respectively. The impact of litter amendment and distance to litter (i.e., soil layer) on extractable MCPA and sum of PLFAs concentrations in soil cores were tested using a linear mixed effect model (R-package 'lme4', Bates et al., 2014) with litter amendment and layer as fixed effects and soil cores as random effect. We used the R-package 'multcomp' (Hothorn et al., 2008) to test for pairwise differences.

3. Results

3.1. The effect of litter on soil respiration and microbial biomass

The mean respiration rate of control soil remained between 0.37 ± 0.04 and $0.74 \pm 0.06 \, \mu mol \, g^{-1} \, d^{-1}$ (Fig. S1). Soil with maize litter amendment had the highest respiration rate ($5.2 \pm 0.2 \, \mu mol \, g^{-1} \, d^{-1}$) at the beginning (day 5) of the experiment. It decreased by a factor of 5 to $0.98 \pm 0.1 \, \mu mol \, g^{-1} \, d^{-1}$ towards the end (Fig. S1). This resulted in $114 \pm 4 \, \mu mol \, g^{-1}$ cumulative CO_2 with litter amendment compared to $37 \pm 1 \, \mu mol \, g^{-1}$ cumulative CO_2 without litter addition after 65 days (Fig. 1). Thus, the mean respiration rate was at maximum 7-fold higher after 5 days ($t_{5.3} = 61.6, p < 0.001$) and cumulative respiration was 3-fold higher after 65 days ($t_{5.7} = 53.1, p < 0.001$) with maize litter addition compared to control. No significant differences between soils amended with unlabeled MCPA and $^{13}C_6$-MCPA were detected.

Without litter amendment, PLFAs and FAs$_{OM}$ were similar in all three soil cores. PLFAs ranged between 32 ± 3.4 and $37 \pm 5.6 \, nmol \, g^{-1}$, whereas FAs$_{OM}$ between 49 ± 3.0 and $61 \pm 0.6 \, nmol \, g^{-1}$ (Fig. 2; see also Table S1). PLFA indicative of Gram-negative and Gram-positive bacteria were equally represented within the living soil microbiome of the control. Maize litter addition boosted tFAs concentration and changed the proportion of living and non-living soil microbiome. In comparison to control, total PLFAs in
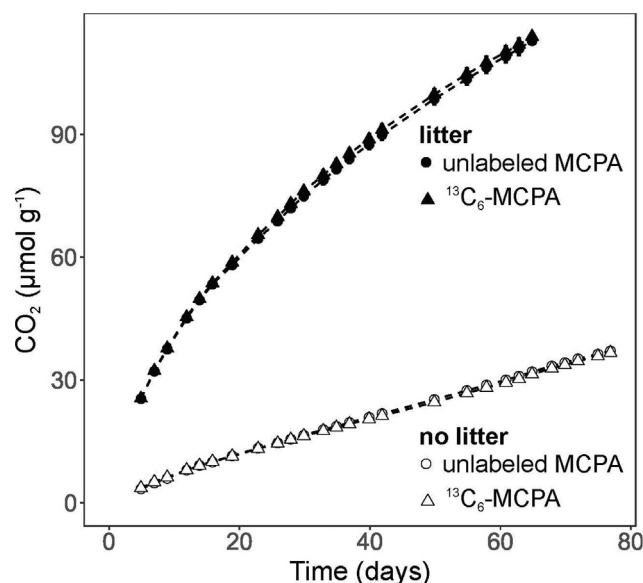

Fig. 1. Cumulative respiration of soil ($\mu mol \, g^{-1}$) treated with unlabeled MCPA and $^{13}C_6$-MCPA with and without litter addition. The respiration is shown as integrated data comprising the three layers over the whole soil column.

detritusphere were 1.6-fold higher ($p < 0.001$), whereas FAs$_{OM}$ were 2.4-fold higher ($p < 0.001$). No significant differences in total amounts of PLFAs were found for transition and bulk soil and FAs$_{OM}$ for bulk soil due to maize litter amendment. In contrast to the control, FAs indicative of Gram-negative bacteria showed an increased contribution of this group within the living soil microbiome (PLFAs) in detritusphere (see Fig. 2A and Table S1A; $p < 0.001$) and non-living microbiome (FAs$_{OM}$) in detritusphere and transition zone with maize litter amendment (Fig. 2B and Table S1B). Litter amendment also resulted in boosted concentrations of fungal PLFA in detritusphere ($p < 0.001$) and of FAs$_{OM}$ in detritusphere and transition zone ($p < 0.001$) in comparison with the control (Fig. 2B; see also Table S1B).

3.2. Mineralization and dissipation of $^{13}C_6$-MCPA

Cumulative mineralization of $^{13}C_6$-MCPA in soil with and without litter expressed as a percentage of the initially added ^{13}C is shown in Fig. 3. Mineralization of $^{13}C_6$-MCPA in control soil was low. It increased slowly during the experiment and reached only $6\% \pm 4.7\%$ of $^{13}C_6$-MCPA equivalents after 77 days. Litter amendment significantly accelerated mineralization of $^{13}C_6$-MCPA following a 20-day lag phase. On day 65, ~7 times more $^{13}C_6$-MCPA was mineralized in the presence of litter than in the control soil ($27\% \pm 3.8\%$ vs. $4.1\% \pm 3.7\%$, $t_{10} = 10.7, p < 0.001$).

No significant differences in the total MCPA-derived C (incl. $^{13}C_6$-MCPA before extraction) and in the extractable MCPA with depth were noticed in the control soil cores (see Fig. 4). In addition, the total MCPA-derived C was almost completely explained by extractable MCPA and in all layers (75–79% vs. 80–85% of initial $^{13}C_6$-MCPA equivalents).

Maize litter addition to soil significantly reduced ($p < 0.001$) the amounts of extractable $^{13}C_6$-MCPA and the total MCPA-derived C (Fig. 4). Total MCPA-derived C was accordingly 88%, 51% and 54% at detritusphere, transition and bulk soil. Extractable $^{13}C_6$-MCPA in the bulk soil with litter decreased to $40\% \pm 8\%$ of the initial $^{13}C_6$-MCPA equivalents. It was even more reduced to $7.7\% \pm 6\%$ and $11\% \pm 7\%$ in the detritusphere and the transition zone ($p < 0.001$), respectively. Like in the control, the majority of total MCPA-derived C (74%) in the bulk soil was extractable as MCPA parent compound. In contrast to the control, only 9% and 22% of total MCPA-derived C could be assigned to
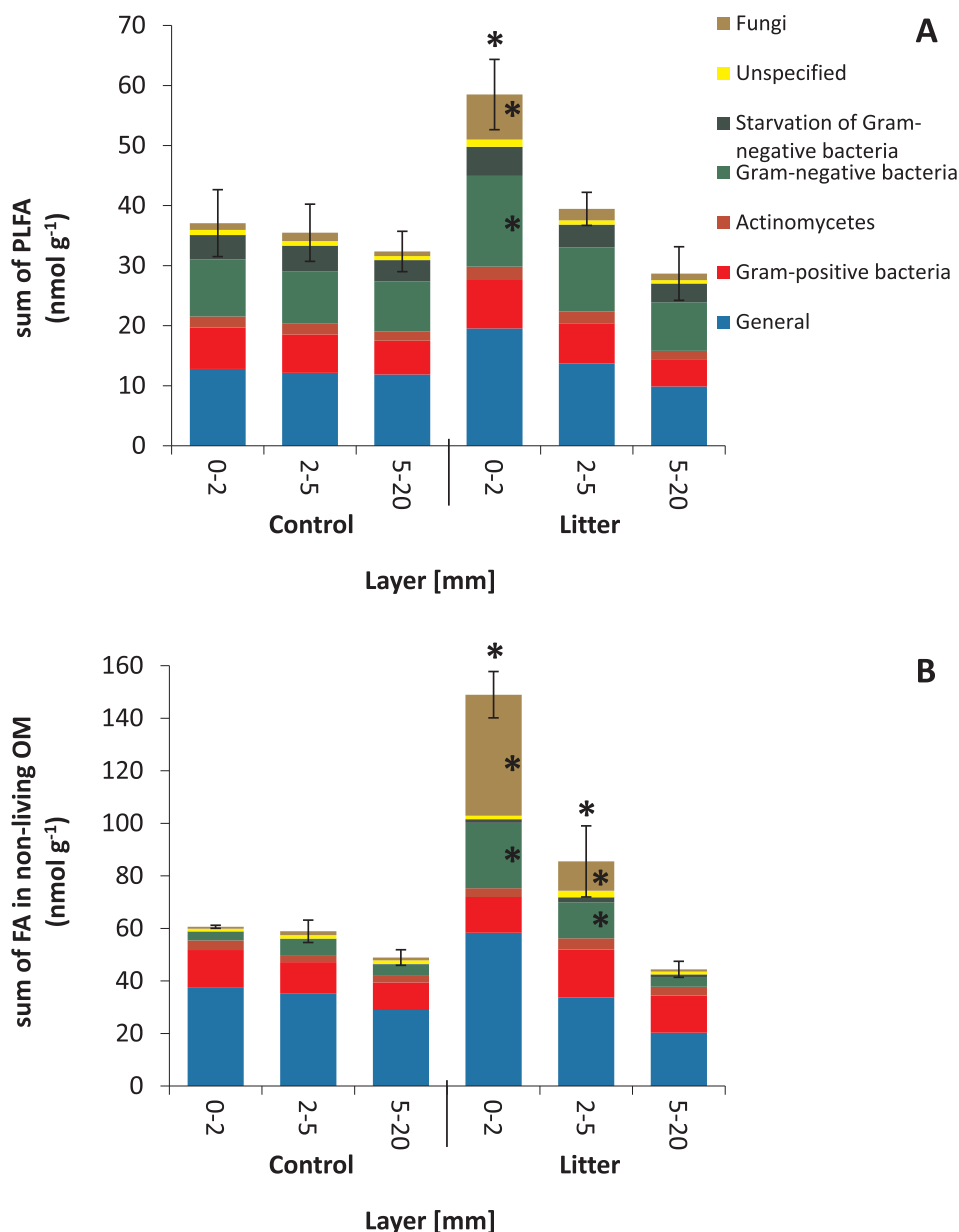
Fig. 2. Sum of PLFAs (A) and of FAsOM (B) in soil amended with $^{13}C_6$-MCPA in different soil layers (nmol g^{-1}): 0–2 mm, 2–5 mm and 5–20 mm. *indicates statistically significant values.

extractable $^{13}C_6$-MCPA in the detritusphere and the transition zone with litter, respectively. In detritusphere and transition zone of litter treatment, the majority of MCPA-derived C was thus represented by the ^{13}C-NERs.

3.3. MCPA-derived ^{13}C in fatty and amino acids

Only slightly higher incorporation of MCPA-derived C into PLFAs in two top layers (0–2 mm and 2–5 mm) than in the bottom layer 5–20 mm was noticed (0.17% and 0.15% vs. 0.10%; see also Fig. 5A, Table S2). Both Gram-positive and Gram-negative bacteria equally incorporated the ^{13}C-MCPA derived label into their PLFAs (Table S2A). Maize litter addition promoted the incorporation of MCPA-derived C into PLFAs in the detritusphere and transition zone ($p < 0.001$). Total ^{13}C-PLFAs were about 3-fold higher in detritusphere and 2.1-fold higher in transition zone of the litter treatment compared to these depths in the control. The PLFA biomarkers indicate that Gram-negative bacteria assimilated the highest amount of ^{13}C-derived MCPA in the two top

layers (Fig. 5A and Table S2A; $p < 0.001$). No significant difference in the contents of ^{13}C-FAs$_{OM}$ between 0 and 2 mm and 2–5 mm of control and of amended treatment was detected (Fig. 5B and Table S2A and S2B). In contrast to the control, significantly higher amounts of ^{13}C in the fungal FAs$_{OM}$ were found in the 0–2 mm and 2–5 mm of the litter-amended treatment (0.063% and 0.052% vs 0.007% and 0.018%; $p < 0.001$).

Without litter amendment, incorporation of MCPA-derived C into total AAs (tAAs) was low at all soil cores. It reached a maximum of 1.2% ± 0.9% in the top layer (Table S3B). The tAAs in the 5–20 mm layer were below the detection limit. Accordingly, incorporation of MCPA-derived C into living biomass AAs (bioAAs) was low (0.08–0.20%, Table S3A) in all soil cores of control. In analogy to ^{13}C-PLFAs, maize litter addition also enhanced the contents of ^{13}C-bioAAs (2-fold) and the contents of ^{13}C-tAAs (15 to 17-fold) in the detritusphere and transition zone ($p < 0.001$) compared with those respective depths in the control (Table S3). The majority of the ^{13}C label in the tAAs (> 71% control soil and > 92% in litter-amended soil)

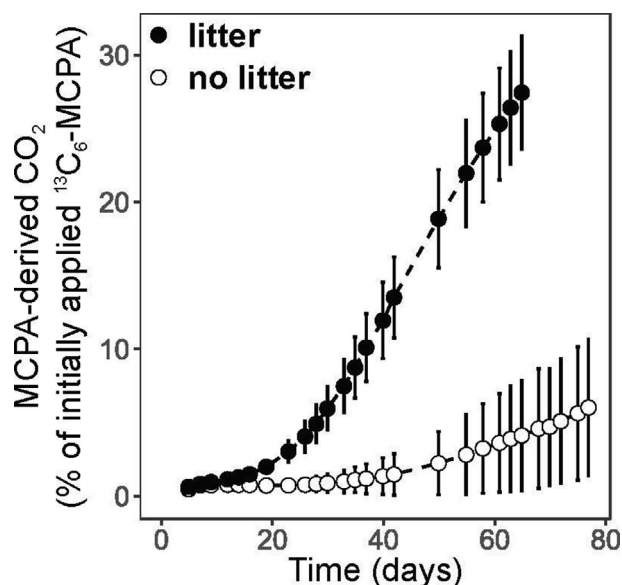**Fig. 3.** Cumulative mineralization of $^{13}C_6$-MCPA in soil with and without litter addition as percentages of the initially applied $^{13}C_6$-MCPA. The cumulative mineralization is shown as integrated data comprising the three layers over the whole soil column.

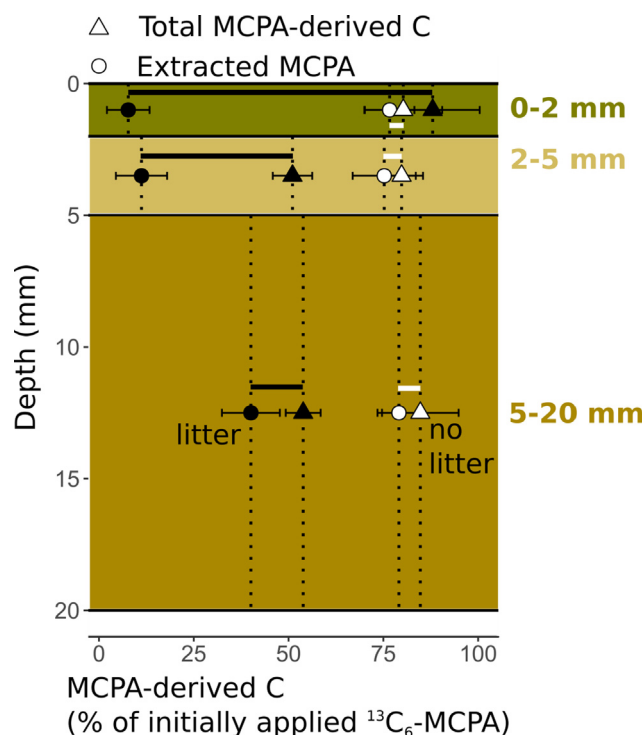**Fig. 4.** The total MCPA-derived C (including $^{13}C_6$-MCPA before extraction) and extractable $^{13}C_6$-MCPA in soil with and without litter addition in different soil layers: 0–2 mm, 2–5 mm and 5–20 mm expressed as percentages of the initially added $^{13}C_6$-MCPA g^{-1} of the respective soil layer. Black markers: litter treatment; white markers: control.

could be assigned to non-living AAs (^{13}C-AAs$_{OM}$; Table S3B) suggesting their stabilization in the OM pool. In contrast to the ^{13}C-bioAAs, the contents of ^{13}C-AAs$_{OM}$ were equally high in the detritusphere and transition zone of the litter-amended treatment, whereas the concentration in the bulk soil was only one fifth of the concentrations in the detritusphere and transition zone (Table S3).

All quantifiable bioAAs were similarly enriched in ^{13}C in the

detritusphere and transition zone of litter treatment. Contrastingly, only four ^{13}C-bioAAs in the bulk soil could be detected (leucine, isoleucine, phenylalanine and lysine; see Table S3A). The distribution pattern of ^{13}C label within the ^{13}C-AAs$_{OM}$ of litter amended soil was in accordance with the distribution pattern of ^{13}C-bioAAs. The AAs carrying most of the label in ^{13}C-AAs$_{OM}$ in the detritusphere were valine, leucine, isoleucine, proline, glutamate and lysine, whereas in the transition zone leucine, isoleucine, proline, aspartate, phenylalanine and lysine dominated the label distribution (see Table S3B).

3.4. Mass balance of $^{13}C_6$-MCPA turnover including bioNER formation

Without litter addition, the majority of extracted $^{13}C_6$-MCPA (~75%) was attributed to the MCPA parent compound at all depths (Fig. 6 and Table S4). Less than 6% $^{13}C_6$-MCPA was identified as NERs, and an even lower percentage could be identified as bioNERs.

Maize litter promoted the formation of total NERs. We found 80.3%, 40% and 13.8% of $^{13}C_6$-MCPA equivalents as NERs in detritusphere, transition zone and bulk soil, respectively (Fig. 6 and Table S4). In the transition zone, the NERs could almost completely be assigned to non-toxic bioNERs (97% of total NERs). In the detritusphere and bulk soil, bioNERs comprised only 46% and 45% of the total NERs.

At the core scale, the majority of initially applied $^{13}C_6$-MCPA in control soil was extracted as MCPA parent compound (77%, Fig. 7, and Table S5) followed by mineralization (6%). Only 4.6% of initially applied $^{13}C_6$-MCPA was non-biogenic NERs. The formation of bioNERs was negligible. In contrast, litter amendment resulted in decreased amounts of extractable parent compound (35% $^{13}C_6$-MCPA) as well as increased mineralization (27% of initially applied $^{13}C_6$-MCPA) and formation of NERs (21% $^{13}C_6$-MCPA). About 65% of the total ^{13}C-NERs were explained by non-toxic bioNERs (14% $^{13}C_6$-MCPA). Total recovery in the mass balances of ^{13}C amounted to 88% of the initial $^{13}C_6$-MCPA equivalents for the control and to 83% for the litter-amended soil (Table S5).

4. Discussion

4.1. Maize litter addition to soil triggered microbial activity

Low respiration, mineralization and dissipation of $^{13}C_6$-MCPA in the control soil compared to the previous studies (Poll et al., 2010, Pagel et al., 2016) might be due to suppressed microbial activity as a result of storage of soil at −20 °C prior to the experiment. This is supported by other studies: while a short and single freeze-thaw of soil did not alter the mineralization of ^{14}C-MCPA (Mortensen and Jacobsen, 2004), prolonged or multiple freezing negatively affected the mineralization kinetics of ^{14}C-MCPA in wetland microcosms (Vandermeeren et al., 2016). Irrespective of the level of microbial activity, the experimental setup allows to clearly reveal the effect of litter addition on MCPA degradation.

Maize litter addition clearly triggered the microbial activity of the soil as reflected by the high initial respiration rate. It tripled cumulative respiration and doubled the sum of tFAs in detritusphere in comparison to 0–2 mm layer in the control soil. A similar effect was observed by Poll et al. (2010) and Pagel et al. (2016) where maize litter placed on top of the mineral soil quadrupled respiration and tripled microbial FAs.

4.2. Maize litter accelerated mineralization and dissipation of MCPA

The accelerated mineralization and dissipation of $^{13}C_6$-MCPA could be related to the increased availability of soluble C which might have been diffused from the maize litter on the top to the transition zone and bulk soil. We found much lower mineralization of $^{13}C_6$-MCPA, both in litter-amended and control soil (27% and 6%) compared to reported values for mineralization of MCPA in the range of 40–70% (Sørensen
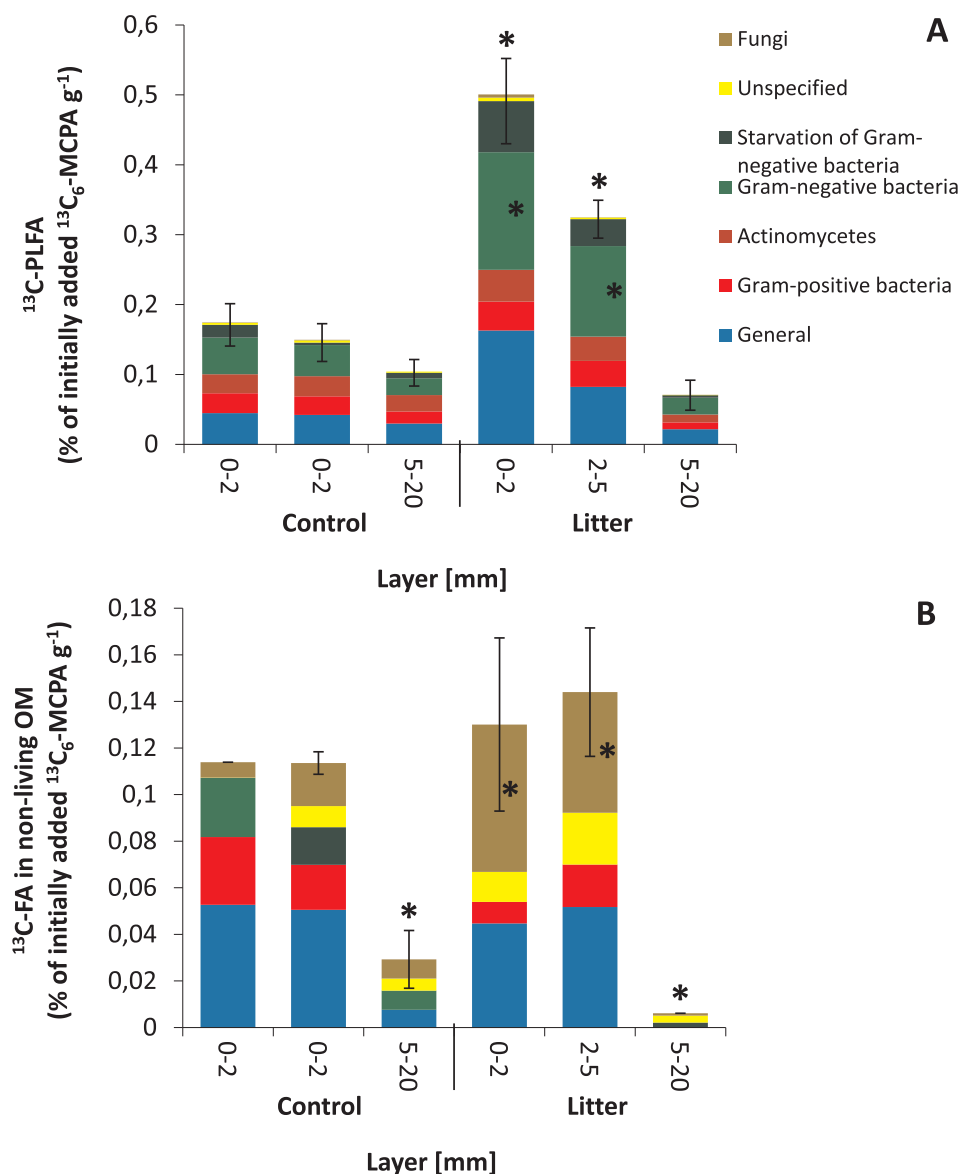
Fig. 5. Distribution of the ^{13}C label within the PLFAs (A) and the FAsOM (B) during biodegradation of $^{13}C_6$-MCPA soil with and without litter addition in different soil layers: 0–2 mm, 2–5 mm and 5–20 mm. The ^{13}C-PLFAs and 13C-FAsOM are given as percentages of the initially applied $^{13}C_6$-MCPA g^{-1} in the respective soil layer. *indicates statistically significant values.

et al., 2006; Jacobsen et al., 2008; Poll et al., 2010; Nielsen et al., 2011; Pagel et al., 2016) and 57% for $^{13}C_6$-2,4-D (Girardi et al., 2013). This divergence might be related to differences in soil type, microbial activity and microbial composition. In addition, mineralization showed a much longer lag phase of about 20 days compared with only 7-day lag phase in study by Poll et al. (2010) or no apparent lag phase by Girardi et al. (2013) and Pagel et al. (2016). The 20-day lag in the mineralization of $^{13}C_6$-MCPA was presumably a combined effect of the time-dependent re-growth of microorganisms after freezing or their adaptation to the high MCPA application rate, which was 10-fold higher than the recommended application rate (5 mg kg^{-1}) of MCPA in agriculture (Saleh et al., 2016).

Along with enhanced mineralization, litter addition also influenced the $^{13}C_6$-MCPA dissipation down to a soil depth of 20 mm, even in the absence of downward advective transport. The amounts of extractable $^{13}C_6$-MCPA in this study (35%) were much higher than the reported < 1% for extractable ^{14}C-MCPA from the litter-amended soil by Poll et al. (2010) and Pagel et al. (2016). In these two studies; however, mineralization of ^{14}C-MCPA was higher and about 7% of the applied

^{14}C was leached from soil. Similar to Poll et al. (2010) and Pagel et al. (2016), we found the lowest residual MCPA concentration in the soil layer closest to litter. Coincidently, we also found highest formation of non-biogenic NERs (43.5%) in the detritusphere. This finding possibly indicates the stabilization of MCPA-C in the form of parent $^{13}C_6$-MCPA or its main transformation product 4-chloro-2-methylphenol. Low amounts of 4-chloro-2-methylphenol are usually measured in soil after aerobic transformation of MCPA as it has a short half-life of 3.55 days (US EPA). We did not analyze 4-chloro-2-methylphenol; however, this transformation product is rapidly converted to CO_2 or sorbed to the solid matrix (Fava et al., 2005). 4-chloro-2-methylphenol has a slightly stronger affinity to OM than MCPA (Fava et al., 2005; Jacobsen et al., 2008; Nielsen et al., 2011; Hiller et al., 2012; López-Piñeiro et al., 2013; Piwowarczyk and Holden, 2013); thus it is likely that this transformation product substantially contributed to non-biogenic NER formation.

4.3. Maize addition stimulated Gram-negative bacteria and fungi

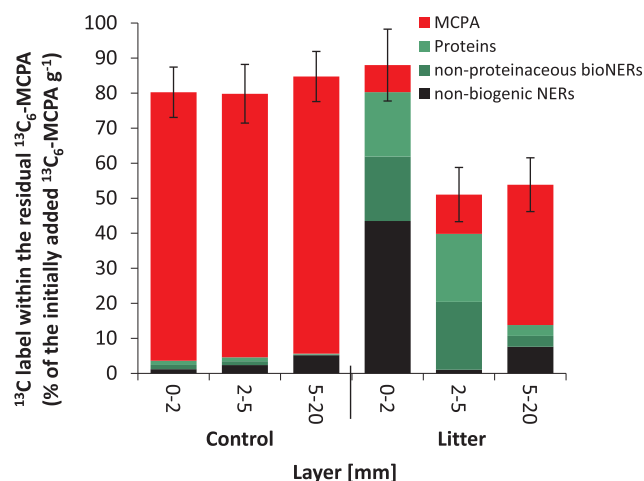Microbial biomass formation and assimilation of ^{13}C-derived MCPA

Fig. 6. Distribution of remaining ^{13}C label on extractable $^{13}C_6$-MCPA and NERs (non-biogenic NERs and bioNERs) in control soil and with litter amendment in different soil layers: 0–2 mm, 2–5 mm and 5–20 mm. Proteins: 13C-tAAs, non-proteinaceous bioNERs: other biomolecules calculated based on known 50% content of proteins (^{13}C-tAAs). The data are presented as percentages of the initially applied $^{13}C_6$-MCPA g^{-1} in the respective soil layer.
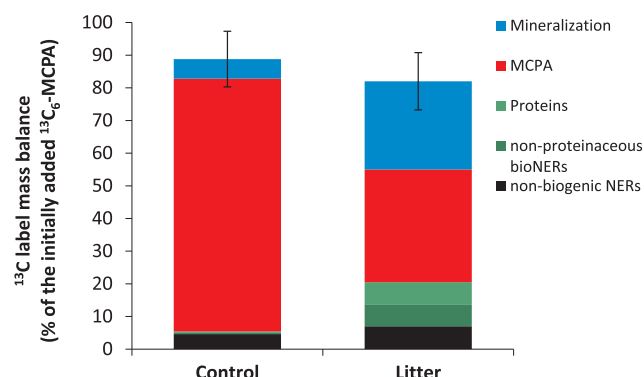

Fig. 7. Distribution of ^{13}C label on mineralization, MCPA, non-biogenic NERs and biogenic NERs during degradation of $^{13}C_6$-MCPA (mass balance) in soil with and without litter amendment. Proteins: 13C-tAAs, non-proteinaceous bioNERs: other biomolecules calculated based on known 50% content of proteins (13C-tAAs). The ^{13}C mass balance of $^{13}C_6$-MCPA is shown as integrated data comprising the three layers and as percentages of the initially applied $^{13}C_6$-MCPA to each soil column.

into the biomass (fatty acids and amino acids) was highest in close proximity to the added maize litter. The addition of fresh C to the soil not only increased the microbial biomass, but also stimulated the incorporation of ^{13}C into Gram-negative bacteria and fungi. The striking contribution of fungi to the non-living soil microbiome (^{12}C-FAs$_{OM}$) and to the non-living $^{13}C_6$-MCPA degraders (^{13}C-FAs$_{OM}$) in our study indicates that transformation of MCPA might have been initiated by fungi in the detritusphere. Evidence on fungal degradation of phenoxyacetic acid herbicides was previously reported by Castillo et al. (2001) and Lerch et al. (2009). This finding is in a good agreement with previous studies that showed increased ergosterol concentrations and abundances of fungal ITS gene fragments during accelerated MCPA degradation in response to litter addition (Poll et al., 2010; Ditterich et al., 2013; Pagel et. al., 2016, Saleh et al., 2016). Fungi are known to play a pivotal role in the initial decomposition of complex organic substrates in soils (de Boer et al., 2005; Strickland and Rousk, 2010). Therefore, we assume that the fungi may have initiated MCPA degradation and supported bacteria in the further conversion of MCPA to CO$_2$. In addition, fungi may have also contributed to the C mobilization and transport of litter derived C into the soil thereby supporting the growth

of bacterial MCPA degraders. Gram-negative bacteria were also the preferential degraders of phenoxyacetic acid herbicide 2.4-D in soil as shown by ^{13}C-stable isotope probing (Lerch et al., 2009; Nowak et al., 2011). This is consistent with our finding that the Gram-negative bacteria were the main utilizers in the later degradation of $^{13}C_6$-MCPA. However, we cannot decide whether the ^{13}C derived MCPA was directly incorporated into the PLFAs of Gram-negative bacteria or indirectly as a result of cross-feeding, e.g. via the fungal biomass. To clarify that, more data on the contents of ^{13}C-PLFAs$_{OM}$ and ^{13}C-FAs$_{OM}$ in time course would be necessary. In addition, labeling pattern of bioAAs and AAs$_{OM}$ with ^{13}C was similar to that observed in the previous soil degradation study with phenoxyacetic acid herbicide $^{13}C_6$-2,4-D (Nowak et al., 2011). This suggests that MCPA degradation could be analogous to the structurally similar 2,4-D.

4.4. Maize litter enhances bioNER and non-biogenic NER formation

Maize litter addition accelerated microbial turnover of $^{13}C_6$-MCPA and also enhanced the formation of proteinaceous bioNERs. The MCPA-derived C in proteins (based on the tAAs hydrolyzed from proteins) in the litter-amended soil was mainly stabilized in the OM pool as proteinaceous bioNERs since only a small proportion was found in the living biomass AAs (< 8% of the ^{13}C in the total AAs). This is in agreement with a previous study where 90% of the ^{13}C-tAAs derived from $^{13}C_6$-2,4-D were stabilized in the non-living OM pool (Nowak et al., 2011).

The NERs in the transition zone of litter-amended treatment were mainly composed of harmless bioNERs which explained 97% of total NERs. In contrast to the transition zone, the bioNERs in the detritusphere and bulk soil of litter-amended soil comprised 46% and 45% of the total NERs, respectively, and thus 54% and 55% of the NERs in these layers was non-biogenic NERs of unknown structural identity. The high contribution of bioNERs to the total NERs in the transition zone of litter-amended treatment is related to an improved nutrients supply from the maize litter placed on top. This, in turn resulted in an increased microbial activity and microbial conversion of MCPA to bioNERs. Contrary to the transition zone, the preferential formation of non-biogenic NERs in the detritusphere could be a result of the sorption of MCPA transformation product 4-chloro-2-methylphenol to solid matrix. Phenolic transformation products of phenoxyacetic acids degradation can be bound to solid matrix by oxidoreductive enzymes, such as laccases and peroxidases via covalent bonding (Bollag et al., 1991, 1992; Hatcher et al., 1993; Dec and Bollag, 1997; Xu and Bhandari, 2003). Oxidoreductive enzymes are commonly produced by fungi (Bollag et al., 1991). A high activity of such enzymes is thus consistent with the high abundance of fungal biomarkers (PLFAs and FAs$_{OM}$) in detritusphere. This thus may explain the high formation of the non-biogenic NERs in the detritusphere which could have been mediated by the fungi. The covalent binding of xenobiotics or their transformation products to solid matrix results in the formation of highly persistent non-biogenic NERs type II with medium or low risk (Kästner et al., 2014). The formation of non-biogenic NERs of type II is still favorable over the physically sequestered NERs type I since it limits the bioavailability of toxic chemical to living organisms and prevents the chemical from the leaching to the waters.

Integrated over the three layers, the ^{13}C-bioNERs in the litter-amended soil amounted only to 14% of $^{13}C_6$-MCPA equivalents and comprised about 65% of the total ^{13}C-NER. In contrast, virtually all NERs could be assigned to bioNERs (amounting to 44% of the initially added ^{13}C) in a previous experiment studying $^{13}C_6$-2,4-D turnover in a different soil (Nowak et al., 2011). The maize litter thus promoted not only microbial activity, degradation of $^{13}C_6$-MCPA and bioNER formation in soil, but also formation of non-biogenic NERs of unknown structural identity which can be potentially toxic.

5. Conclusions

This is the first ^{13}C isotope labeling study that shows the effect of plant litter-soluble C on pesticide degradation at a fine mm-depth scale of soil. Maize litter accelerates microbial activity, mineralization of MCPA and NER formation in soil. However, our results highlight strong differences in the formation of harmless bioNERs and potentially toxic non-biogenic NERs at the mm-scale. Beyond the detritusphere, this finding might have important consequences for NER formation in other highly active soil microhabitats with high C input (e.g., the rhizo-sphere). Although plant litter addition to soil can also increase the formation of non-biogenic NERs, it is still favorable since it promotes the degradation of organic chemicals and reduces the extractable fraction of the parent compound, i.e. the most mobile and available pool. However, the type of non-biogenic NERs (type I versus type II) formed in response to additional C input need to be analyzed in detail. In particular, increased formation of physically sequestered NERs (Type I) carries the risk of delayed remobilization, which might turn soils from sinks into sources of pollutants.

CRediT authorship contribution statement

Karolina M. Nowak: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Anja Miltner:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - original draft, Writing - review & editing. **Christian Poll:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing - review & editing. **Ellen Kandeler:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing - review & editing. **Thilo Streck:** Funding acquisition, Resources, Supervision, Writing - review & editing. **Holger Pagel:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.envint.2020.105867.

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